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13. ABSTRACT *(Maximum 200 Words)*

Angiogenic vessel growth in solid tumors is crucial for the survival of the tumor and provides an avenue for the tumor cells to metastasize to distant sites. Breast cancer tumors are also dependent on angiogenesis for survival. Therefore, understanding the underlying mechanisms of the angiogenic process in breast cancer will enhance our knowledge of the carcinogenic process and provide potential therapeutic targets for the treatment of breast cancer. Our lab has previously shown that FGF-BP1, a binding protein for the angiogenic factor FGF-2, is overexpressed in a certain subset of breast cancers. In the current study we have found that FGF-BP1 is also overexpressed in the mammary tumors of the Min/+ mice, a mouse model that expresses high levels of the oncogene beta-catenin. Using *in vitro* assays we also show that FGF-BP1 is a direct transcriptional target of beta-catenin that may be working through a novel 13bp fragment in the FGF-BP1 promoter.

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INTRODUCTION

Breast cancer is dependent on angiogenesis for growth and malignant progression. Without angiogenesis, the tumors would not have the proper nutrients for growth and would be limited in their ability to enter the circulatory system and metastasize [1]. The process of angiogenesis is controlled and regulated by a number of different protein factors. FGF-2 is one of the most potent of the pro-angiogenic factors [2]. FGF-2 is normally sequestered in the extracellular matrix (ECM) and can be released by enzymatic cleavage of heparin sulfate proteoglycans or by the fibroblast growth factor binding protein (FGF-BP) [3]. FGF-BP can reversibly bind to FGF-2 and release it from the ECM [3]. Previous work from our laboratory indicate that FGF-BP acts as an angiogenic factor and is expressed in a subset of breast cancers as well as in squamous cell carcinoma and colon carcinoma as indicated by histological analysis of human tumor samples (Cabal et. al. unpublished data). We examined the expression of FGF-BP in the mammary tumors of the Min/+ mouse. This mouse model has a germline mutation in the adenomatous polyposis coli (APC) gene. A second somatic hit in the second allele of this gene produces a non-functional APC protein [4]. This mutation results in overexpression of the oncogene beta-catenin. In this study we examine the regulation of FGF-BP1 by beta-catenin in breast cancer.

BODY

We have previously shown that FGF-BP is expressed in the mammary tumors of the Min/+ mouse. In this report work accomplished toward the goals stated in Aim 2 (To study the mechanism of regulation of FGF-BP by the APC/Beta-catenin signaling pathway in breast cancer) are discussed. First, it was necessary to confirm that FGF-BP was indeed regulated by

beta-catenin. Although FGF-BP was coexpressed with beta-catenin in mammary tumors of the Min/+ mouse, this only confirms spatial correlation of the two proteins. In order to establish a direct relationship between beta-catenin and FGF-BP, a series of *in vitro* assays were undertaken using two different breast cancer cell lines. The MDA-MB-468 breast cancer cell line expresses endogenous FGF-BP and low levels of beta-catenin. This cell line was used to test whether increases in beta-catenin levels would effect the endogenous expression of FGF-BP. To induce higher levels of beta-catenin, the MDA-MB-468 cells were treated with lithium chloride. Lithium inhibits glycogen synthase kinase-3beta (GSK-3beta) a negative regulator of beta-catenin. Upon treatment of MDA-MB-468 with Lithium chloride, which resulted in a subsequent increase of cytoplasmic and nuclear beta-catenin, there was a 3-fold induction of FGF-BP1 mRNA levels (Figure 1). Therefore, beta-catenin can regulate the endogenous FGF-BP1 gene product in breast cancer cell lines.

As stated in Aim 2- experimental series #2, the possibility that beta-catenin regulates FGF-BP at the transcriptional level has been examined. To determine whether FGF-BP regulation by beta-catenin occurs at the promoter, we transiently co-transfected the SK-BR-3 breast cancer cell line with a 1Kb portion of the FGF-BP promoter (-1060/+62 FGF-BP-luciferase) and a wild-type beta-catenin expression vector (Figure 2). We found that beta-catenin is able to activate the promoter up to 3.5 fold in breast cancer cells (Figure 2). Furthermore, E-cadherin, which sequesters beta-catenin, was co-transfected with FGF-BP and beta-catenin and was able to reverse the beta-catenin-mediated induction of FGF-BP (Figure 2). These results verify that FGF-BP is a direct target of beta-catenin and that it occurs at a transcriptional level. Many beta-catenin target genes are transcriptionally regulated via a TCF-site (5'-A/T A/T CAAAG-3') [5]. In order to determine the relevant promoter regions of FGF-BP for beta-

catenin regulation, a series of FGF-BP promoter fragments were cloned into the PGL3 empty vector. These fragments were sequential 5'-deletions of the promoter beginning from a 1 Kb piece. Some of these constructs were created using restriction enzyme digestions while the others were made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). A schematic of the FGF-BP promoter with relevant site is provided in figure 3a. These progressive 5' deletion constructs of FGF-BP +/- beta-catenin were transiently transfected into the SK-BR-3 cell line (Figure 3b). Deletion of two sites, located at -545 and –167, with homology to TCF did not effect the beta-catenin induction of the promoter (Figure 3b). However, beta-catenin induction of FGF-BP was lost between –152 and –139 of the promoter (Figure 3b). This 13-base pair region does not have homology to TCF. It is possible that beta-catenin utilizes a novel transcription factor site for its modulation of FGF-BP transcription.

Figure 1. Lithium Chloride (LiCl) induction of FGF-BP1 mRNA in the MDA-MB-468 cell line. *(A)* MDA-MB-468 cells were plated in 10 cm tissue culture dishes at 70% density in IMEM+10%FBS for 24 hrs. at 37^0C before treatment. Following 24 hr. incubation, media was replaced with fresh IMEM+10%FBS plus NaCl/inositol (50mM/10mM) or LiCl/inositol (50mM/10mM) for 16hrs. RNA was isolated and analyzed by northern blot (see *methods*) for FGF-BP1 expression. Cytoplasmic levels of β-catenin were measured by western blot analysis (α-tubulin was used as a loading control). *(B)* Levels of FGF-BP1 mRNA were quantified by Phosphorimager (Stratagene) and corrected for loading by GAPDH mRNA levels. The addition of LiCl resulted in a 3-fold induction of FGF-BP1 mRNA levels as compared to control samples (NaCl). Values are representative of three separate experiments (mean+/- SE). Each experimental parameter was done in duplicates within each experiment. The asterix (*) indicates a statistically significant difference between the LiCl treatment and NaCl treatement as determined by student-T test ($p<0.05$).

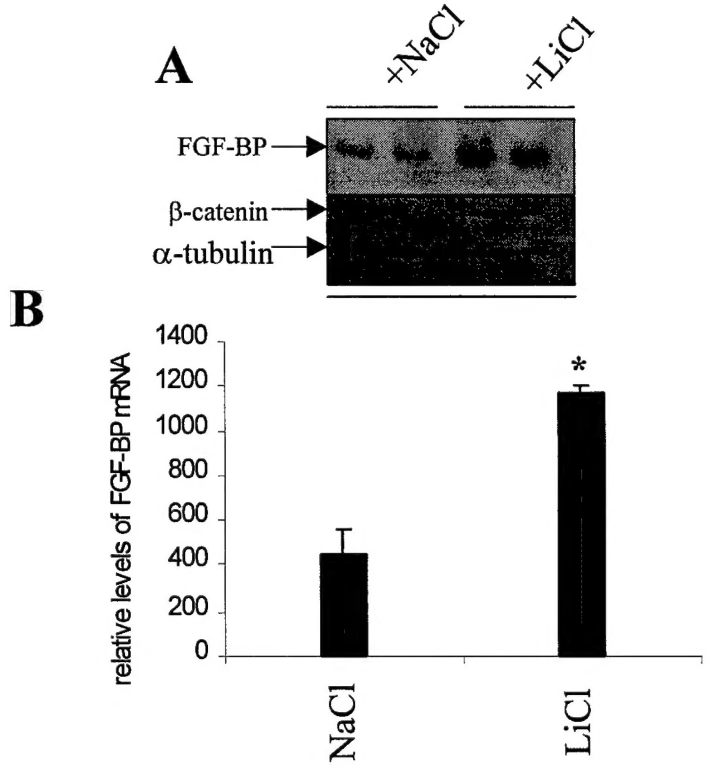
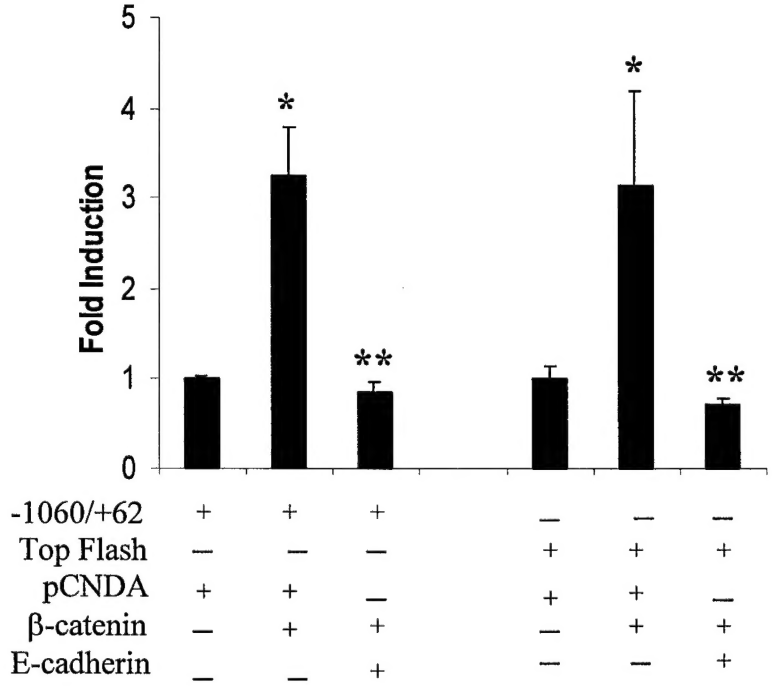

A
+NaCl
+LiCl
FGF-BP
β-catenin
α-tubulin
B
relative levels of FGF-BP mRNA
1400
1200
1000
800
600
400
200
0
*
NaCl
LiCl

Figure 1

Figure 2. β-catenin induction of FGF-BP1 promoter activity in the SK-BR-3 cell line. SK-BR-3 cells were transiently transfected using the Fugene (Roche) transfection system. The cells were plated at 70% confluence in 12-well plates and transfected 24 hrs. later. The cells were then incubated for 48 hrs. at which time FGF-BP-luciferase activity was measured. Luciferase is expressed as fold induction of β-catenin-transfected over pCDNA-transfected (control) cells for each construct (FGF-BP and Topflash, a promoter vector with multimerized TCF/LEF transcriptional factor sites). Transfection efficiency was corrected for by TK-renilla. In the SK-BR-3 cells β-catenin induces the FGF-BP1 promoter approximately 3-fold. E-cadherin was co-transfected to sequester β-catenin and, subsequently, reverse β-catenin induction of FGF-BP promoter activity. Values represent the mean +/- S.E. from three different experiments. . The asterix (*) indicates a statistically significant difference between the –1060/+62 FGF-BP1-luciferase construct cotranfected with β-catenin and the –1060/+62 FGF-BP1-luciferase construct contransfected with the control vector pCDNA as determined by student-T test ($p<0.05$). The double asterix (**) indicates a statistically significant difference between the –1060/+62 FGF-BP1-luciferase construct cotranfected with β-catenin and E-cadherin and the –1060/+62 FGF-BP1-luciferase construct contransfected with β-catenin as determined by student-T test ($p<0.05$).


Fold Induction
5
4
3
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-1060/+62 + + + — — —
Top Flash — — — + + +
pCNDA + + — + + —
β-catenin — + + — + +
E-cadherin — — + — — +

Figure 2

Figure 3a. Schematic of FGf-BP1 promoter. 5'-deletion mutants were made at –417,–152,-139, and –129 (see methods). The –417 and –152 deletion constructs removed to potential TCF/LEF transcription factor binding sites. Important transcription factor binding sites required for promoter activity are also indicated.

Figure 3b. Effect of sequential 5' deletions on β-catenin induction of the FGF-BP1 promoter. Each construct was co-transfected with 0.75 μg of either pCDNA or β-catenin using the Fugene (Roche) transfection system for 48 hours. PGL3-basic (Promega) is the empty vector used for cloning of the FGF-BP constructs. These constructs were transiently transfected into the SKBR3 cell line as described in figure 14. The most significant loss of activity in β-catenin induced FGF-BP promoter activity occurs in the 13 base region of –152 and –139 (a reduction from approximately 6-fold to less than 2-fold). This region has sequence homology to REL1, an NFkB family member.

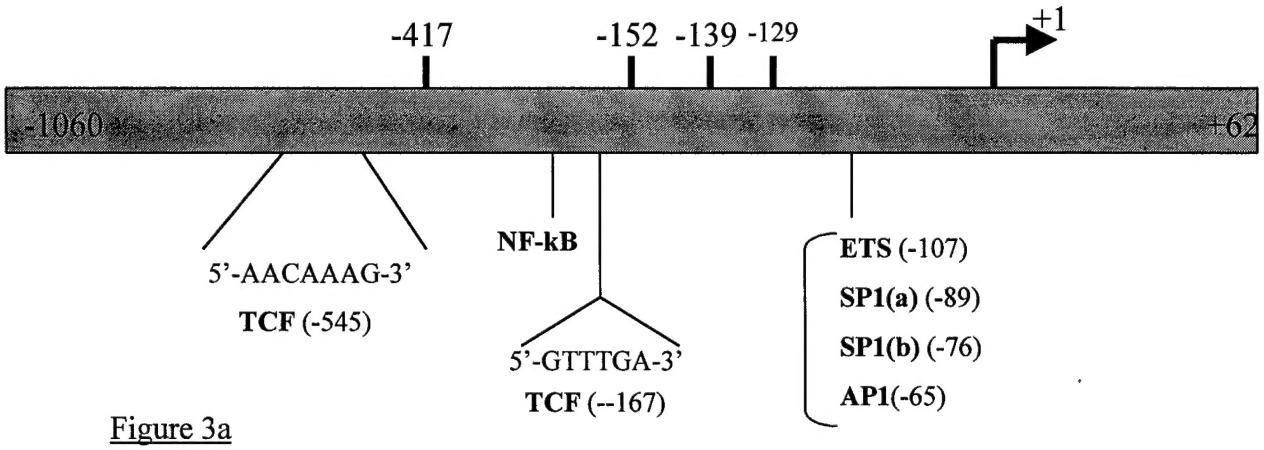

Figure 3a

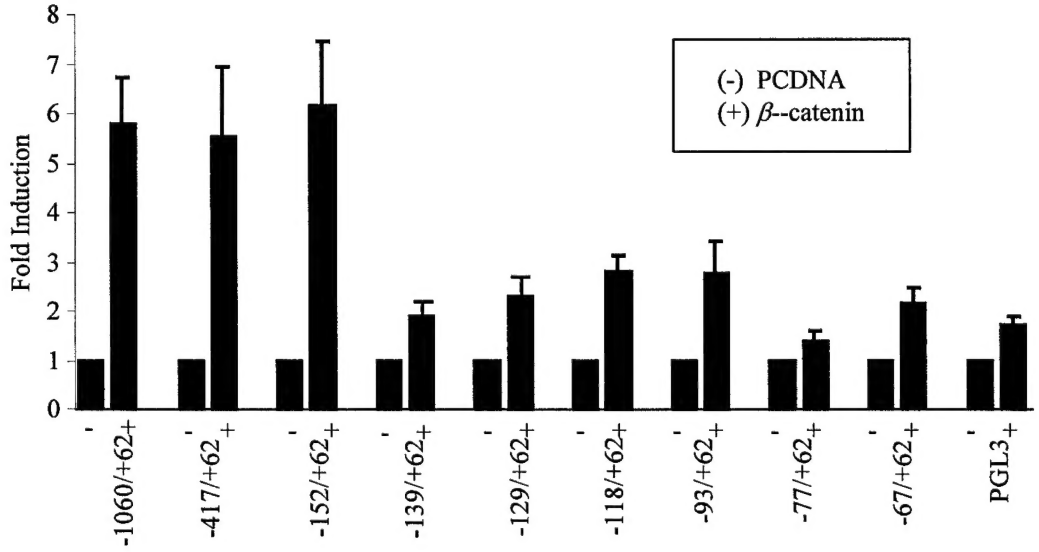

Figure 3b

Research Accomplishments

- Identification of a relevant genetically defined mouse model of breast cancer to study the expression pattern and function of the angiogenic factor FGF-BP.
- We have identified FGF-BP as a novel target gene of the WNT/beta-catenin signaling pathway.

Abstracts

Ray, R., Cabal-Manzano, R., Riegel, AT., Wellstein, A. The Angiogenic Factor Fibroblast Growth Factor Binding Protein (FGF-BP), a novel beta-catenin target gene. American Association of Cancer Research, New Orleans, LA (2001).

Conclusions

The goal of **Aim 2, experimental series #2** was to determine if FGF-BP1 is a target gene of the WNT/beta-catenin signaling pathway in breast cancer. Using a variety of *in vitro* techniques (transient transfections, northern blot analysis, western blot analysis, site-directed mutagenesis, etc.) we have confirmed that beta-catenin does regulate FGF-BP. Because beta-catenin is overexpressed in human breast cancer [6] it may possibly act as an oncogene in this disease as it does in colon cancer. Our work presents the possibility that beta-catenin may influence tumor angiogenesis in breast cancer through FGF-BP. Additionally, we have identified a 13 base pair region of the FGF-BP promoter that is necessary for beta-catenin induction of FGF-BP transcription. This site has not been identified in other beta-catenin target genes and may be a novel transcription factor site for beta-catenin-promoter interactions.

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